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Involvement of *Arabidopsis thaliana* phospholipase D ζ 2 in root hydrotropism through the suppression of root gravitropism

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Abstract

Root hydrotropism is the phenomenon of directional root growth toward moisture under water-deficient conditions. Although physiological and genetic studies have revealed the involvement of the root cap in the sensing of moisture gradients, and those of auxin and abscisic acid (ABA) in the signal transduction for asymmetric root elongation, the overall mechanism of root hydrotropism is still unclear. We found that the promoter activity of the *Arabidopsis* phospholipase D ζ 2 gene (*PLD ζ 2*) was localized to epidermal cells in the distal root elongation zone and lateral root cap cells adjacent to them, and that exogenous ABA enhanced the activity and extended its area to the entire root cap. Although *pld ζ 2* mutant root caps did not exhibit a morphological phenotype in either the absence or presence of exogenous ABA, the inhibitory effect of ABA on gravitropism, which was significant in wild-type roots, was not observed in *pld ζ 2* mutant roots. In root hydrotropism experiments, *pld ζ 2* mutations significantly retarded or disturbed root hydrotropic responses. A drought condition similar to that used in a hydrotropism experiment enhanced the *PLD ζ 2* promoter activity in the root cap, as did exogenous ABA. These results suggest that *PLD ζ 2* responds to drought through ABA signaling in the root cap and accelerates root hydrotropism through the suppression of root gravitropism.

Keywords ABA - Drought - Phospholipase D - Root cap - Root gravitropism - Root hydrotropism

Abbreviations

ABA	Absciscic acid
ACC	Aminocyclopropane-1-carboxylic acid
BA	Benzyl adenine
DAG	Day after germination
GA	Gibberellic acid
GUS	β -Glucuronidase
IAA	Indole acetic acid
MeJA	Methyl jasmonic acid
PLD	Phospholipase D

Introduction

Water deficiency is a frequently occurring difficulty for plants growing in natural fields. To survive this adversity, land plants are equipped with various means of altering their metabolism, morphology, and developmental processes (Maseda and Fernandez 2006; Seki et al. 2007; Shinozaki and Yamaguchi-Shinozaki 2007; Shao et al. 2008). Of these, root hydrotropism, directional growth of roots toward moisture, is the most active means that plants can exert in the early stages of water deficiency. Root hydrotropism has been described for many plant species, including peas and maize (Takahashi 1997; Eapen et al. 2005; Takahashi et al. 2009). During the root hydrotropic response, the root cap sense a moisture gradient and transfer the signal to the root cell elongation zone, where asymmetric cell elongation results in root curvature (Jaffe et al. 1985; Takahashi and Scott 1993; Takano et al. 1995; Hirasawa et al. 1997). The involvement of calcium ions, auxin, and abscisic acid (ABA) in the signalling for this response has been revealed by genetic and physiological studies (Takahashi and Suge. 1991; Takano et al. 1997; Mizuno et al. 2002; Takahashi et al. 2002; Eapen et al. 2003; Kaneyasu et al. 2007; Ponce et al. 2008). From *Arabidopsis thaliana*, mutants specific to this response have been obtained (Eapen et al. 2003; Kobayashi et al. 2007; Miyazawa et al. 2009). Moreover, in *Arabidopsis*, water deficiency is supposed to suppress root gravitropism to prioritize root hydrotropism (Takahashi et al. 2003; Eapen et al. 2005; Takahashi et al. 2009). However, the mechanisms are still unclear, not only for the establishment of root hydrotropism, but also the suppression of root gravitropism under water-deficient conditions.

Phospholipase D (PLD) is the enzyme that hydrolyzes glycerophospholipids such as phosphatidylcholine to produce phosphatidic acid. Animal PLDs are frequently activated in response to extracellular stimuli and implicated in intracellular signaling pathways for cell survival and proliferation, cytoskeletal reorganization, vesicular trafficking, and oxidative bursts (Jenkins and Frohman 2005; Cazzolli et al. 2006; Oude Weernink et al. 2007). Also in plants, which encode plant-specific-type PLDs in addition to eukaryote-general-type PLDs (Qin and Wang 2002), PLDs are known to be involved in cellular responses to biotic and abiotic stresses, including drought (Testerink and Munnik 2005; Wang 2005; Bargmann and Munnik 2006; Wang et al. 2006; Xue et al. 2007; Li et al. 2009). *PLD ζ 2*, one of two eukaryote-general-type PLD genes in *Arabidopsis*, responds to phosphate starvation and exogenous auxin at the transcription level, and is

thought to function in phosphate recycling and auxin-mediated phenomena including root gravitropism (Cruz-Ramirez et al. 2006; Li et al. 2006a, b; Li and Xue 2007). However, the loss of its function does not cause critical defects in either development under normal growth conditions or survival against phosphate starvation, whereas it confers hyposensitivity to exogenous auxin, a retarded root gravitropic response, and precocious root meristem exhaustion during phosphate starvation compared to the wild type (Cruz-Ramirez et al. 2006; Li and Xue 2007). To better understand the biological function of *PLD ζ 2*, we focused on its function in the root tip region, and investigated its involvement in root gravitropic and hydrotropic responses.

Materials and methods

Plant material and growth conditions

All *Arabidopsis thaliana* (L.) Heynh lines used were of the Columbia ecotype, and Columbia was used as the wild type. The T-DNA insertion lines, SALK_094369 and GABI_123E01, were identified in the collection of the Salk Institute Genomic Analysis Laboratory (Alonso et al. 2003) and GABI-Kat at Bielefeld University (Rosso et al. 2003), respectively. *Arabidopsis* plants were grown on 0.8% agar medium containing Murashige and Skoog salts, B5 vitamins, and 1% sucrose under continuous light at 23°C, unless otherwise noted. Phytohormone treatments for histochemical analysis were performed by transferring seedlings grown for 7 days after germination (DAG) on the normal agar medium to agar medium supplemented with 1 or 10 μ M ABA, 1 or 10 μ M benzyl adenine (BA), 10 μ M indole acetic acid (IAA), 10 μ M gibberellic acid (GA), 10 μ M aminocyclopropane-1-carboxylic acid (ACC), or 100 μ M methyl jasmonic acid (MeJA).

Transgene constructs and transgenic *Arabidopsis* plants

To construct the reporter gene in which the *PLD ζ 2* promoter directs the expression of the *Escherichia coli* β -glucuronidase (GUS), a 1,408-bp DNA fragment encompassing the region between the termination codon of the upstream neighboring gene and the *PLD ζ 2* initiation codon, was cloned into the binary vector pBI101 at the multi-cloning site. The

sequences of upstream and downstream junctions were 5'-CCTGCAGACCGGTTTCATGGTTTTGTGG-3' and 5'-CTTCACGACAAGCAAGAACAGGGATCC-3', respectively (the underlined sequences are the *Sse*8387I and *Bam*HI restriction sites, respectively, of pBI101). Strains of *Agrobacterium tumefaciens* LBA4404 carrying each construct were used to transform *Arabidopsis* by vacuum infiltration. The resulting transgenic plants were self-pollinated, and T3 homozygous plants were used for analysis.

Histochemical GUS analysis

Histochemical GUS analysis was performed using the basic procedure described by Jefferson et al. (1987). Whole seedlings or organs were submerged in cold 90% acetone for 20 min at 4°C. After several washes in 100mM sodium-phosphate buffer (pH 7.4), they were incubated for appropriate periods at 37°C in a solution containing 0.5 mg/ml 5-bromo-4-chloro-3-indoryl- β -D-glucuronide, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 100 mM sodium phosphate (pH 7.4). The reaction was stopped by several washes in the sodium-phosphate buffer, and plant pigments were removed with 70% ethanol. Cross-sectioning was performed according to the instructions for Technovit 7100 (Heraeus Kulzer GmbH, Wehrhelm, Germany).

Confocal laser scanning microscopy

The fluorescence of propidium iodide was observed with a confocal scanner unit CSU10 (Yokogawa Denshikiki Co., Ltd., Tokyo, Japan) and a CCD camera ORCA-ER-C4742-80 (Hamamatsu Photonics, Hamamatsu, Japan) on an Axiovert 100M apparatus (Carl Zeiss, Wetzlar, Germany). IPLab version 3.71 (BD Biosciences Bioimaging, Rockville, MD, USA) was used for image processing.

Root gravitropism analysis

Seedlings grown for 7 DAG on a vertically standing 1 % agar medium were transferred to another vertically standing medium with or without 1 μ M ABA and grown for 1 day. Then, the medium was rotated by 90°, and the root images were recorded at appropriate

times. The angle of root curvature was measured on the image with the aid of the image-analysis program Image-Ana LITE (Omron, Kyoto, Japan).

Root hydrotropism analysis

Root hydrotropism analyses were performed as previously described (Takahashi et al. 2002). In the experiment with a closed chamber, 1% agar medium was placed in a square plastic dish (14 cm x 10 cm x 1 cm), and a portion of the 1-cm-wide edge of the dish was removed. Seedlings at 7 DAG were transferred to the agar plate such that approximately 0.5 mm of each root tip was hanging from the agar plate edge. The plate with seedlings was glued to the vertical inner surface of an acrylic chamber (24 cm x 22 cm x 16 cm), in which 500 ml of a saturated solution of K_2CO_3 was placed at the bottom. In the experiment with a combined agar plate, two pieces of 1% agar medium without or with 0.4 M sorbitol, which reduces water potential by approximately 1 MPa, were placed in the upper left or lower right half of a square plastic dish (14 cm x 10 cm x 1 cm), respectively, so that they contacted each other along a diagonal line (Supplemental Fig. S3b). Seedlings at 7 DAG were transferred to the upper left medium with their root tips touching the borderline. The plate was placed vertically at 23°C in the dark.

Results

ABA enhanced the *PLDζ2* promoter activity in root caps

We performed detailed histochemical analyses of the *PLDζ2* promoter activity using a reporter gene containing the 1,408-bp intergenic DNA fragment upstream from the *PLDζ2* initiation codon and the GUS-coding fragment. Under normal growth conditions, transgenic plants with the reporter gene exhibited GUS activity in pollen grains, root tips, and root vascular tissues (Fig 1a-e) as previously reported (Cruz-Ramirez et al. 2006; Li and Xue 2007). Intense staining was often, but not reproducibly, observed in cotyledons and leaves (Fig. 1a). Cross sections of root tips revealed that GUS activity was located relatively weakly to the epidermal cells of the distal elongation zone, and relatively strongly to the lateral root cap cells adjacent to them (Fig. 1f, g). This cell-type specific

localization of the *PLD ζ 2* promoter activity in the root tip has not been previously described. Then, we focused on the activity in the root tip and investigated its responses to phytohormone signals. Of the chemicals we examined, 1 μ M ABA enhanced the activity and extended the active region to more distal parts of the lateral root cap and columella root cap cells (Fig. 1h). In contrast, 1 μ M BA suppressed the activity in the root tip (Fig. 1i). Note that either treatment, even at a higher concentration, did not result in significant changes in tissues other than in the root tip (Supplemental Fig. S1). Other chemicals, IAA, GA, ACC, and MeJA, did not affect promoter activity in the root tip, while IAA activated the promoter in leaves and the root vascular system as previously reported (Supplemental Fig. S1; Li and Xue 2007).

No morphological abnormality was observed in *pld ζ 2* mutant root caps

The results of the promoter analysis suggested that *PLD ζ 2* functions in the root cap, especially in the presence of an ABA signal. To investigate such functions, we first compared root cap morphology between the wild type and the *pld ζ 2* mutant, SALK_094369, which is thought to be a null allele (Cruz-Ramirez et al. 2006; Li et al. 2006a, b), using confocal laser scanning microscopy after staining with propidium iodide. However, we could not detect a phenotypic change in the morphology of mutant root caps, in either the absence or presence of exogenous ABA. In the normal agar medium, both wild-type and mutant root caps were approximately 0.5 mm in length, and covered a varied number of elongating cells in each epidermal cell file (Supplemental Fig. S2). In the agar medium with 1 μ M ABA, they showed basically the same morphology as those in the absence of exogenous ABA, while they were reduced in length, along with a size reduction in the root meristematic zone (Supplemental Fig. S2). This suggests that *PLD ζ 2* has no critical role in the morphological development of the root cap.

The inhibitory effect of ABA on gravitropism was affected in *pld ζ 2* mutant roots

Lateral root cap cells constitute the major auxin path for root gravitropism from the columella root cap, where gravity changes are sensed and auxin is asymmetrically distributed, to the root cell elongation zone, where the asymmetric inhibition of cell elongation by auxin results in root curvature (Ottenschlager et al. 2003; Swarup et al.

2005). Studies have reported that the root gravitropic response of the *pldζ2* mutant is retarded compared with that of wild-type roots (Li and Xue 2007) and that exogenous ABA affects gravitropism in wild-type roots (Han et al. 2009). Hence, we next examined root gravitropism in the *pldζ2* mutant in the presence of exogenous ABA. Both the negative effects of the *pldζ2* mutation and exogenous ABA on root gravitropism were observed also in our experimental system (Fig. 2). However, in the presence of 1 μM ABA, the gravitropic responses of *pldζ2* mutant roots were not significantly affected, and resulted in a similar responsiveness to that of wild-type roots (Fig. 2). These results indicate that *PLDζ2* is involved in ABA signaling that suppresses root gravitropism.

Root hydrotropism was affected in *pldζ2* mutant roots

ABA has been reported as a positive factor for root hydrotropism in *Arabidopsis* (Takahashi et al. 2002; Eapen et al. 2003; Ponce et al. 2008) and proposed as a signal antagonizing the root gravitropic response in hydrotropic responsive roots (Eapen et al. 2005). Hence, we hypothesized that *PLDζ2* functions in root hydrotropism through the ABA signaling that suppresses root gravitropism. To obtain evidence supporting this hypothesis, two types of experiments for root hydrotropic responses were performed. A water potential gradient was constructed between an agar plate and a saturated salt solution in a closed chamber for one experiment (Fig. 3a, b), and between normal and sorbitol-containing media in a combined agar plate for another (Supplemental Fig. S3). In the experiment with a closed chamber, the *pldζ2* mutant exhibited a significantly retarded hydrotropic response compared to the wild type (Fig. 3a, b). A similar condition to this experiment, in which only a root tip was exposed to drought, activated the *PLDζ2* promoter as exogenous ABA did (Fig. 1j, Supplemental Fig. S1), suggesting that drought stress enhances the *PLDζ2* expression through an ABA signal during root hydrotropism. Also, in the combined agar plate, the *pldζ2* mutation disturbed the root tropism repulsive against sorbitol (Supplemental Fig. S3). Another *pldζ2* mutant allele, GABI_123E01, showed similar defects in both experiments (Fig. 3a, Supplemental Fig. S3a). These results indicate that *PLDζ2* contributes to quick and effective hydrotropic responses in *Arabidopsis* roots.

Discussion

PLDζ2 is known to have a positive function in root gravitropism through the sensitization of auxin signaling (Li and Xue 2007). The gene might exert this positive function in the epidermal cells of the distal elongation zone and the lateral root cap cells adjacent to them, where the promoter is active under normal growth conditions. Since these cells correspond to the auxin path for root gravitropism (Ottenschlager et al. 2003; Swarup et al. 2005), this idea is consistent with the former proposition that *PLDζ2* enhances auxin polar transport through intracellular membrane trafficking (Li and Xue 2007). Adding to the positive function, we revealed *PLDζ2* involvement in the ABA signaling that suppresses root gravitropism. Among the possible mechanisms for this involvement, it is most likely that *PLDζ2* acts downstream of the ABA signal, since the *PLDζ2* promoter is activated by ABA in the root cap, and PLDs have frequently been reported to mediate ABA signals (Richie and Gilroy 1998; Jacob et al. 1999; Zhang et al. 2004). Although the molecular mechanism of this suppression is totally unknown, the fact that the ABA treatment and the *pldζ2* mutation did not cause an additive effect with each other on root gravitropism indicates a close relation between ABA signal and *PLDζ2* function in suppressing root gravitropism. Since the *pldζ2* mutation did not affect the root cap structure in either the absence or presence of exogenous ABA, the function of *PLDζ2* in root gravitropism are likely to be independent of the developmental processes of the root cap. Ectopic overexpression of *PLDζ2* has been reported to enhance root gravitropism (Li and Xue 2007). In the overexpressor plants, the ABA-related suppressive function of *PLDζ2* might be overcome by its positive function through auxin signaling. Alternatively, the suppressive function might require the ABA-responsive functions of other genes.

We also revealed *PLDζ2* involvement in root hydrotropism. Because local drought treatment of a root tip activated the *PLDζ2* promoter in the root cap as exogenous ABA did, and because *PLDζ2* is involved in ABA signaling that suppresses root gravitropism, the gene most likely contributes to root hydrotropism through the suppression of root gravitropism. However, *PLDζ2* may also contribute to root hydrotropism through a common auxin signal pathway with root gravitropism, since both tropisms in *Arabidopsis* require auxin responsiveness (Kaneyasu et al. 2007). Recently, a genetic study revealed that the *Arabidopsis* ARF-GEF gene, *GNOM*, is involved in root

hydrotropism (Miyazawa et al. 2009). GNOM is a known regulatory factor of intracellular membrane trafficking, especially for the cycling of auxin transporters between the plasma membrane and inner membrane compartments, and hence, its function is required for auxin-related phenomena, including gravitropism (Steinmann et al. 1999; Geldner et al. 2003). Although *Arabidopsis* root hydrotropism is possibly independent of an active polar transport of auxin (Kaneyasu et al. 2007), PLD ζ 2 and GNOM might act in the same auxin signal pathway that regulates asymmetric cell elongation during both root gravitropic and hydrotropic responses.

Effects of the *pld ζ 2* mutations on root gravitropism and hydrotropism were significant, but not striking, suggesting that *PLD ζ 2* and other PLD genes are functionally redundant. Since protein structure is similar between the two eukaryote-general-type PLDs in *Arabidopsis*, PLD ζ 1 and PLD ζ 2 (Qin and Wang 2002), *PLD ζ 1* possibly has such a redundant function while its involvement in root tropisms has not been examined. It was recently shown that PLD activity originated from multiple PLD genes, including *PLD α 1* and *PLD δ* , were induced upon dehydration in *Arabidopsis* (Bargmann et al. 2009). Those PLDs might also redundantly function in the signal transduction for root hydrotropism. Comprehensive studies using multiple mutants and overexpressors of PLD genes would be helpful to understand overall PLD functions in root hydrotropism. Under natural drought conditions, where plants compete with each other for water escaping from soil, even a subtle delay in root hydrotropism is thought to be fatal. Although such conditions would be difficult to be produced experimentally, they might cause a striking difference in survival between *pld ζ 2* mutants and the wild type.

It is very likely, but still speculative, that the suppression of root gravitropism is a major contribution of *PLD ζ 2* to root hydrotropism. However, the drought response of its promoter in the root cap and the retardation of root hydrotropism in its mutant strongly suggest that the drought-induced expression of *PLD ζ 2* accelerates root hydrotropic responses. This *PLD ζ 2* function, as a signal transducer of drought stress, is presumably critical for plant survival in natural fields, where water deficiency is frequently a fatal problem for plants.

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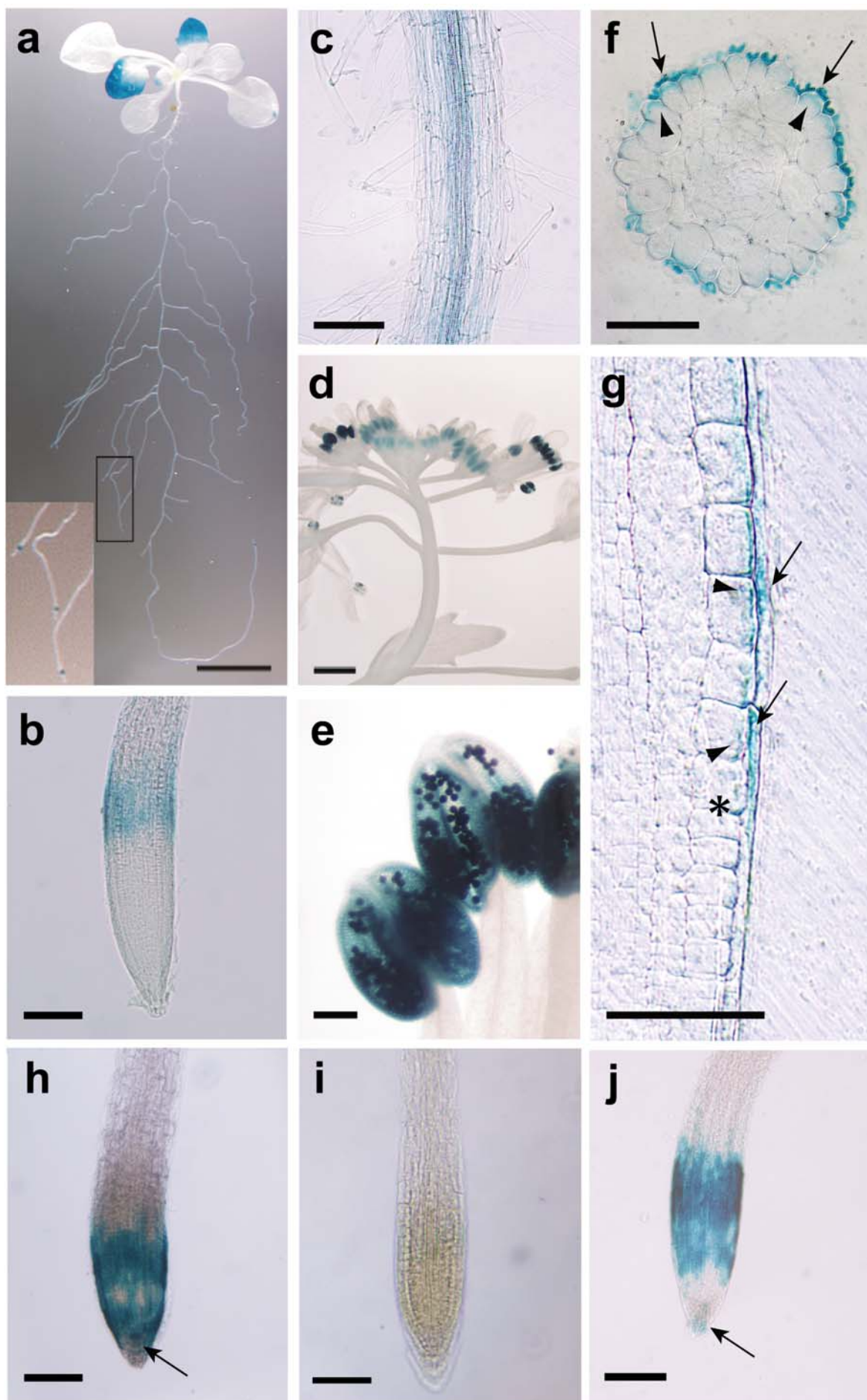
Legends to figures

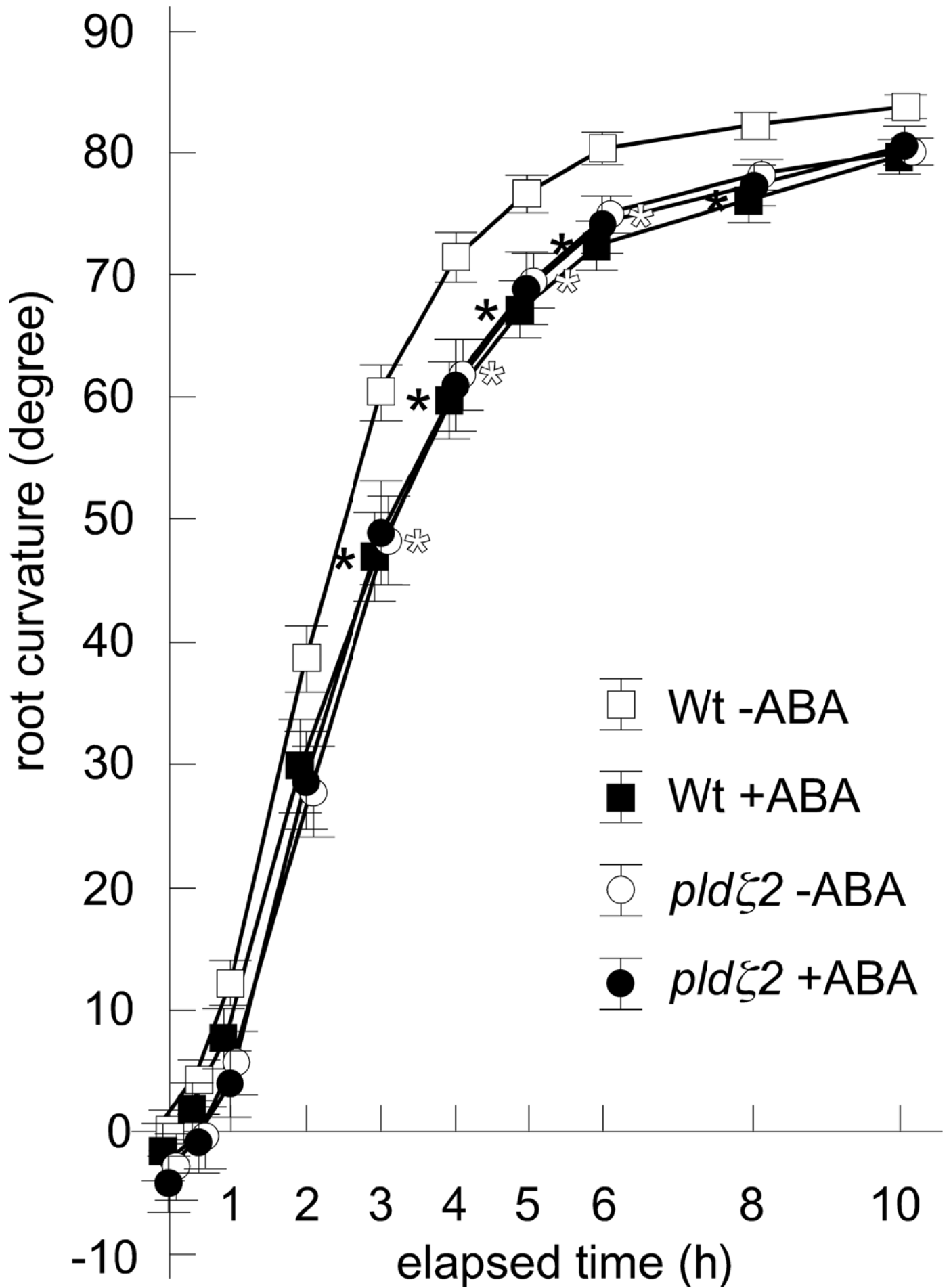
Fig. 1 Histochemical analysis of the *PLDζ2* promoter under various growth conditions. The *PLDζ2* promoter activity was histochemically analyzed using transgenic plants carrying the *pPLDζ2-GUS* gene. **a-g** Plants were grown under normal growth conditions. Seedling at 10 DAG (**a**), main root tip of a 10 DAG seedling (**b**), upper part of a main root at 10 DAG (**c**), inflorescence of a mature plant (**d**), mature anthers (**e**), transverse section of a root in the transition zone (**f**), longitudinal section of a root in the transition zone (**g**). **h-j** Main root tips are shown. Seedlings at 7 DAG were transferred to agar medium containing 1 μM ABA (**h**) or 1 μM BA (**i**) and grown for 1 day, or transferred to an agar plate in a closed chamber for a hydrotropism experiment (see Materials and methods) with approximately 3 mm of the main root tip hanging from the agar plate edge so that the root tip was exposed to drought conditions and grown for 1 day (**j**). An inset in **a** shows a magnified picture of the part encompassed by the square. Arrows and arrowheads indicate the lateral root cap and epidermal cells that exhibit GUS activity, respectively, in **f** and **g**. An asterisk indicates the lower boundary of elongating epidermal cells in **g**. Arrows in **h** and **j** indicate the columella root cap cells that exhibit GUS activity. Bar = 5 mm (**a**), 0.1 mm (**b, c, e, h, i, j**), 1 mm (**d**), and 0.05 mm (**f, g**)

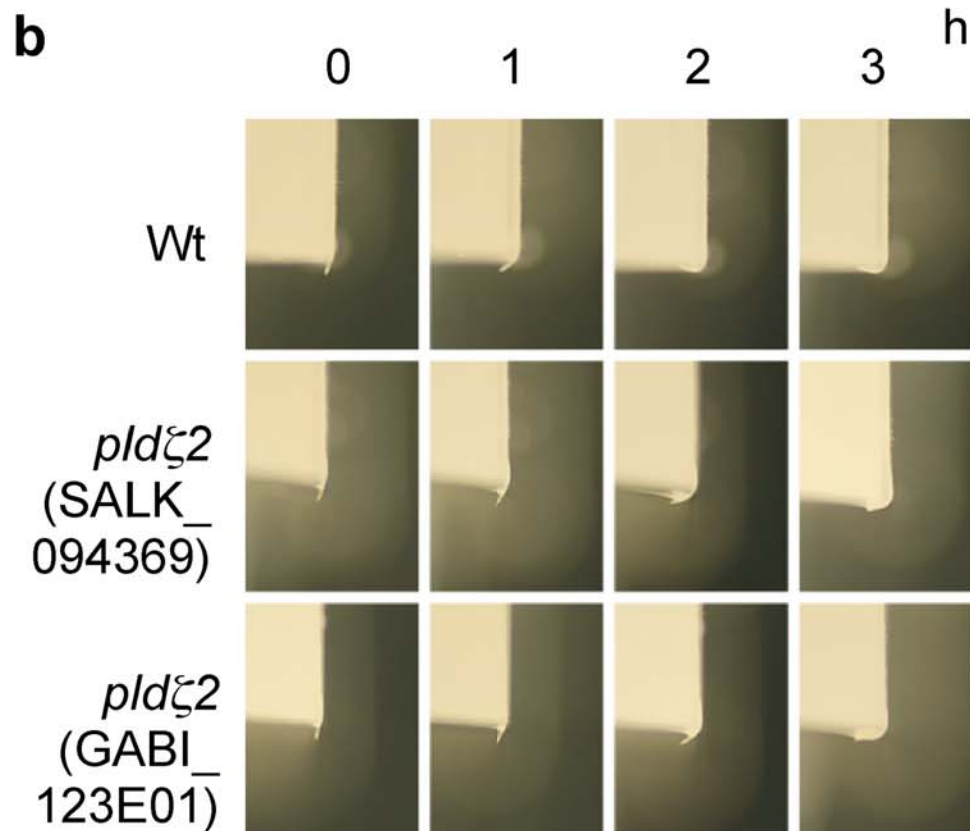
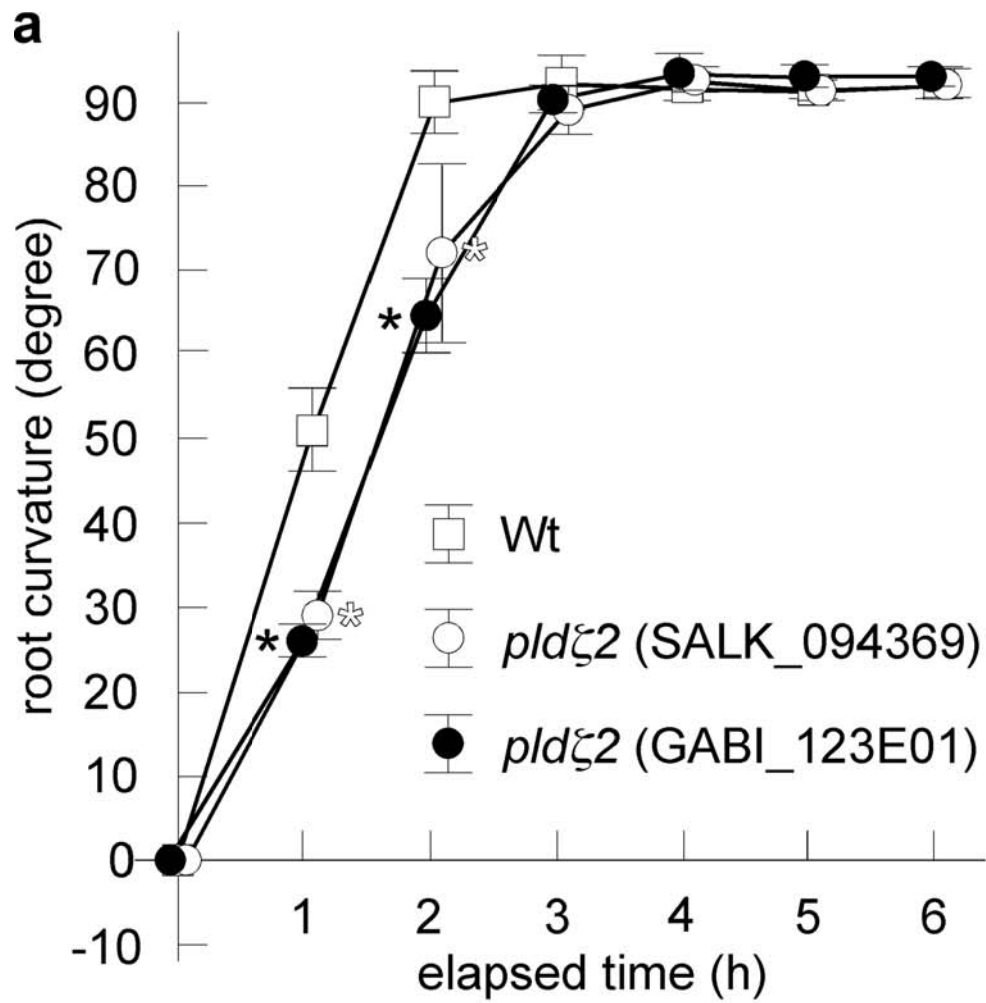
Fig. 2 Gravitropism analysis of *pldζ2* mutant roots in the presence of exogenous ABA. Wild-type and *pldζ2* mutant seedlings at 7 DAG grown on a vertically standing 1 % agar plate were transferred to another vertical agar plate without (-ABA) or with (+ABA) 1 μM ABA. After 1 day's growth, the plate was rotated by 90°. The angles of root curvature were measured at indicated times after rotation, and mean values were plotted. Error bars represent SE ($n = 30$), and statistical analysis was performed using Student's *t*-test. Open and filled asterisks indicate significant differences of mutant values without ABA and wild-type values with ABA, respectively, from wild-type control values without ABA at $P < 0.01$

Fig. 3 Hydrotropism analysis of *pldζ2* mutant roots with a closed chamber. Root hydrotropism of *pldζ2* mutants (SALK_094369 and GABI_123E01) was analyzed in an experiment with a closed chamber. Seedlings at 7 DAG were transferred to an agar plate

in a closed chamber, where the water potential gradient was constructed between the agar plate and a saturated K_2CO_3 solution, such that approximately 0.5 mm of each root tip was hanging from the agar plate edge. **a** The angles of root curvature were measured every hour after transfer, and mean values were plotted. Error bars represent SE ($n = 4$), and statistical analysis was performed using Student's *t*-test. Open and filled asterisks indicate significant differences of SALK_094369 and GABI_123E01 mutant values, respectively, from the wild-type control at $P < 0.01$. The same tendency was observed in three independent experiments. **b** Wild-type and mutant root tips 0, 1, 2, and 3 h after transfer are shown







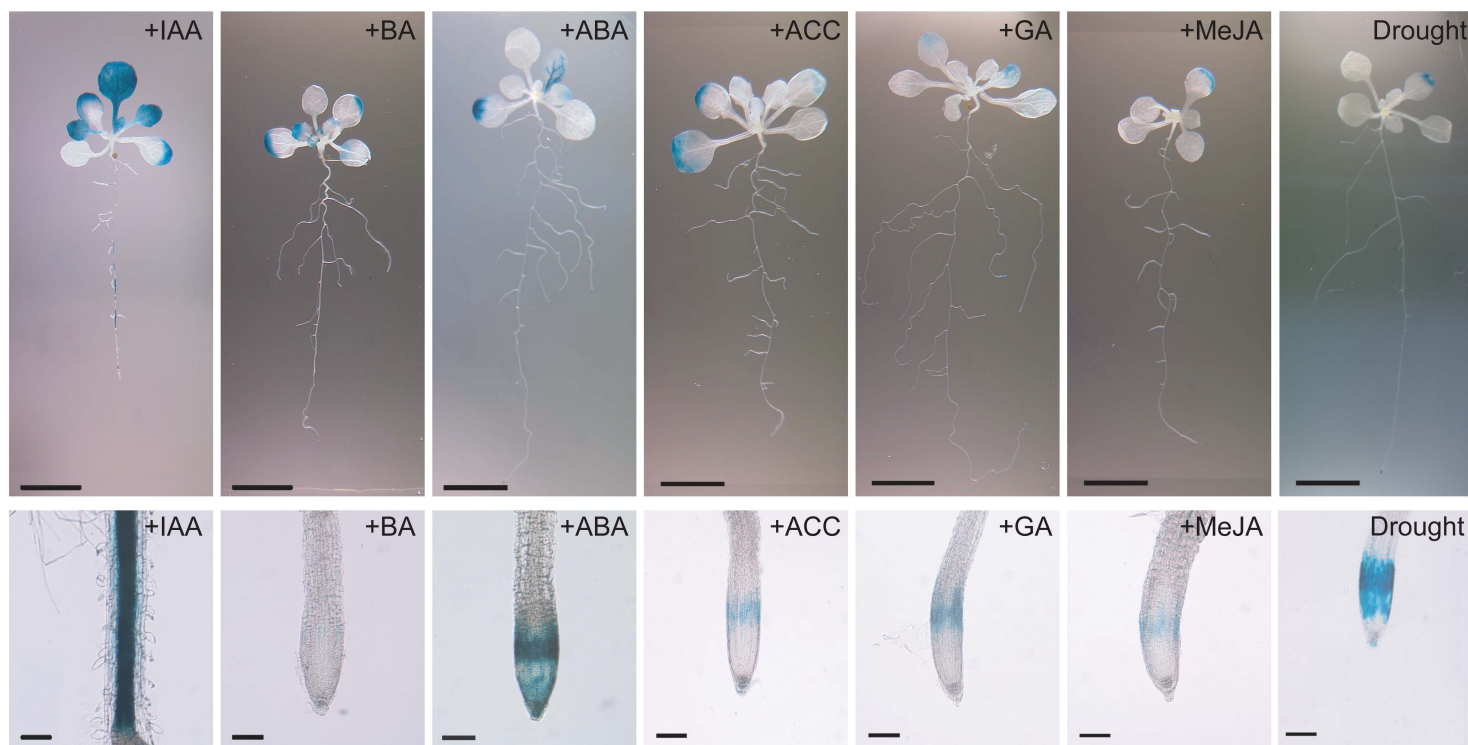


Fig. S1 Histochemical analysis of *PLDζ2* promoter under phytohormone-supplemented and local drought conditions. Seedlings carrying the *PLDζ2-GUS* gene at 7 DAG were transferred to agar medium containing 10 μ M IAA, 10 μ M BA, 10 μ M ABA, 10 μ M ACC, 10 μ M GA, or 100 μ M MeJA, and grown for 3 days, or transferred to an agar plate in a closed chamber for a hydrotropism experiment (see Materials and methods) with approximately 3 mm of the main root tip hanging from the agar plate edge so that the root tip was exposed to drought conditions and grown for 1 day. GUS staining patterns of a seedling (upper row) and a main root tip (lower row) are shown for each condition, except the IAA-supplemented condition, for which an upper part of a main root is shown. Bar = 5 mm (for seedlings), and 0.1 mm (for root tips and an upper part of the root)

Fig.S2

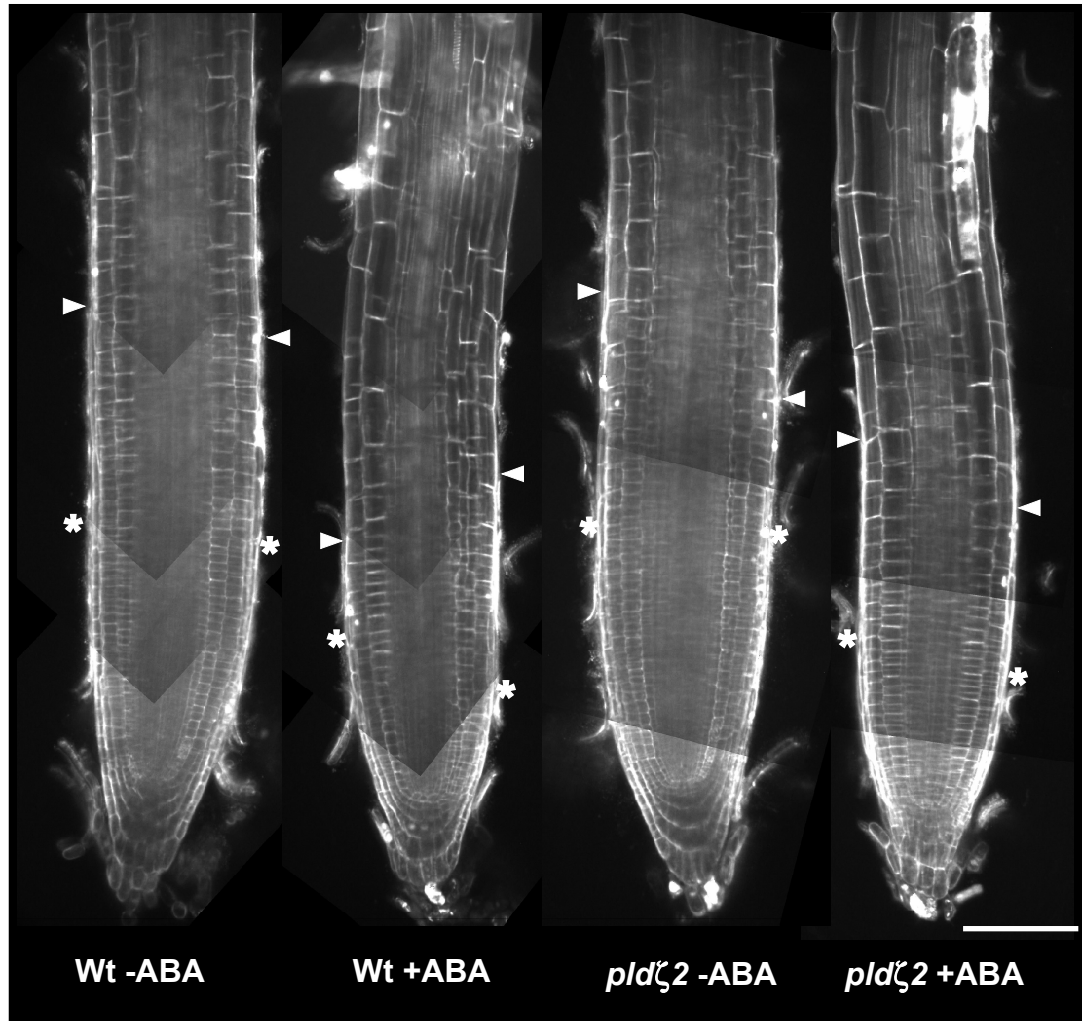


Fig. S2 Morphological analysis of *pldζ2* mutant root caps. Wild-type and *pldζ2* mutant (SALK_094369) seedlings at 7 DAG were transferred to agar medium without (-ABA) or with (+ABA) 1 μ M ABA, and grown for 3 days. The root tip structure was observed using a confocal laser scanning microscopy after staining with propidium iodide. Asterisks indicate the lower boundary of elongating epidermal cells. Arrowheads indicate the upper root cap edge. Bar = 0.1 mm

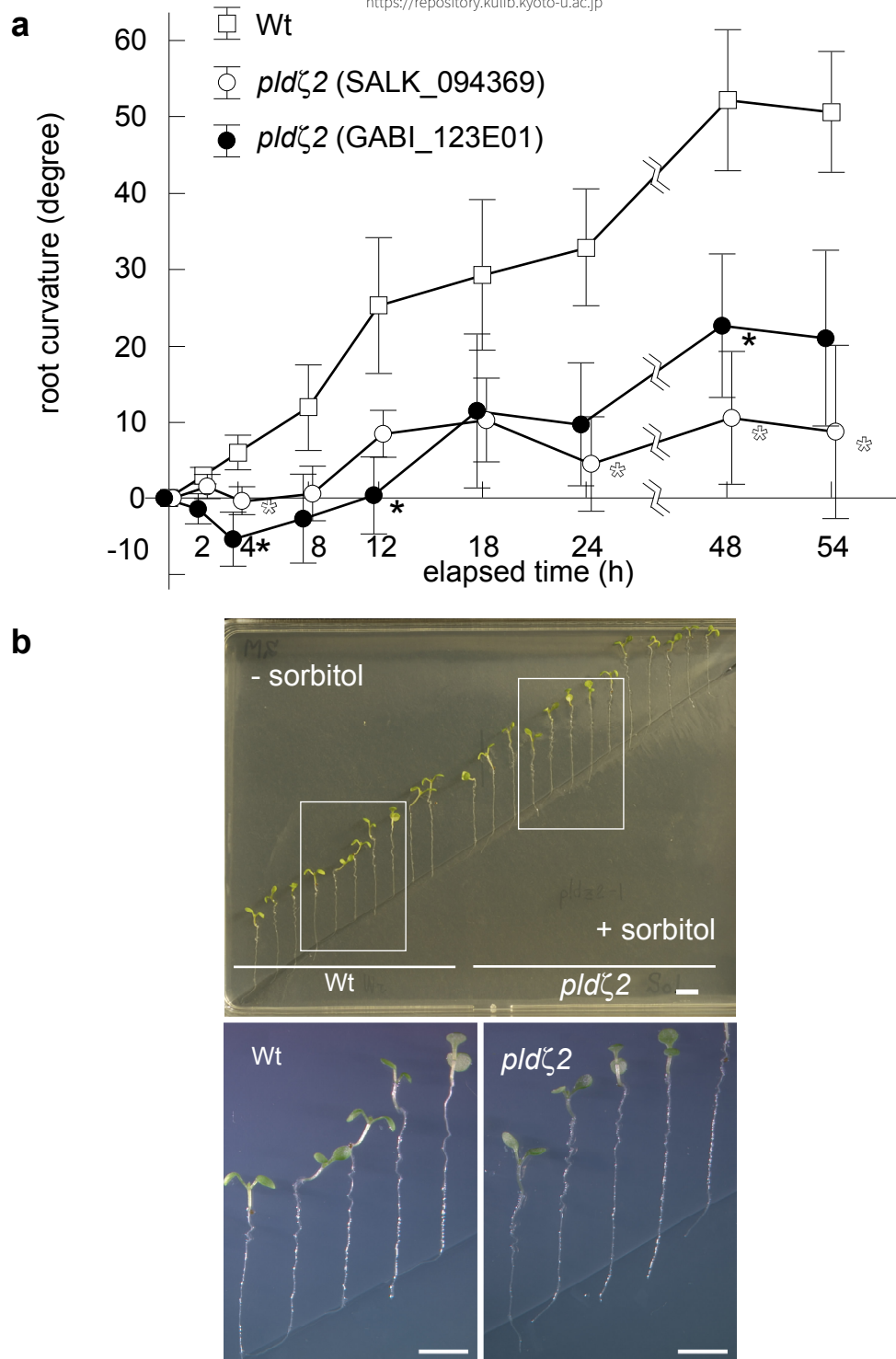


Fig. S3 Hydrotropism analysis of *pld*ζ2 mutant roots with a combined agar plate. Seedlings at 7 DAG were transferred to an agar plate, where the water potential gradient was constructed between 1% agar media without and with 0.4 M sorbitol placed in the upper left or lower right half of a square plastic dish, respectively, such that the main root tips touched the diagonal border line. The plate was placed vertically at 23°C in the dark. **a** The curvature angles of wild-type and *pld*ζ2 mutant (SALK_094369 and GABI_123E01) roots were measured at indicated times after transfer, and mean values were plotted. Error bars represent SE ($n > 8$), and statistical analysis was performed using Student's *t*-test. Open and filled asterisks indicate significant differences of SALK_094369 and GABI_123E01 mutant values, respectively, from the wild-type control at $P < 0.05$. **b** Wild-type and mutant (SALK_094369) seedlings grown on a combined agar plate for 54 h after transfer (upper panel). Magnified pictures of the wild-type (lower left panel) and mutant (lower right panel) seedlings that are enclosed by white squares in the upper panel. Bar = 5 mm